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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Selenium is essential for male fertility. In mature mammalian spermatozoa it is largely restricted to the midpiece harbouring the helix of mitochondria embedded into a keratine-like selenium-enriched matrix called the mitochondrial capsule (1). Selenium deficiency is associated with impaired sperm motility, structural alterations of the midpiece up to breakages, and loss of *flagellum* (2-5). The predominant selenoprotein of the mammalian male reproductive system, phospholipid hydroperoxide glutathione peroxidase (PHGPx), was shown to be preferentially expressed in round spermatids but was hardly detectable in terms of messenger RNA or activity in spermatozoa (6). Here we report that PHGPx persists in spermatozoa but as insoluble, enzymatically inactive material forming the mitochondrial capsule. PHGPx activity of this material can be restored by high concentrations of thiols. PHGPx, thus, acts as a peroxidase in the proliferating germ epithelium to prevent oxidative damage. In the late stages of sperm maturation it is oxidatively cross-linked to become a structural element indispensible for sperm function.

Routine preparations of rat sperm mitochondrial capsules (1) yielded a fraction which was insoluble in 1% SDS and 0.2 mM DTT and displayed expected vesicular appearance in electron microscopy (Fig. 1 a). The vesicles readily disintegrated upon exposure to 0.1 M mercaptoethanol (Fig. 1 b) and became fully soluble in 6 M guanidine-HCL. When the solubilized capsule material was subjected to gel electrophoresis essentially four bands in the 20 kDa region were detected (Fig. 1 c, left lane). Western blotting revealed that the most prominent one reacted with antibodies directed against PHGPx (Fig. 1 c, right lane) which is undetectable as active peroxidase in mature spermatozoa (Tab. 1). Also, N-terminal sequencing of the 21 kDa band representing about 46% of total protein content according to Coomassie stain revealed that it consisted of at least 95% pure PHGPx. Puzzled by this unexpected finding, we investigated the composition of the mitochondrial capsules in more detail by 2D-electrophoresis (Fig. 2 a) followed by microsequencing and/or MALDI-TOF for identification (Fig. 2 b). For this purpose the capsules were dissolved completely in a buffer designed for electrophoretic separation of membrane proteins (see Methods). The spot migrating with an apparent molecular weight of about 21 kDa and focussing at a pH near 8 (spot 3) proved to be PHGPx according to the masses of tryptic peptides detected by MALDI-TOF (Fig. 2 b). By the same technique, also the slightly more acidic charge isomer (spot 4), the more basic ones (spots 1, 2 and 5) as well as the spots 6 and 7 exhibiting a smaller apparent molecular mass were shown to contain PHGPx (Fig. 2 c). The predicted N-terminal (pos. 3-12) and C-terminal peptides (pos. 165-170), the fragment corresponding to positions 100-105 and those expected from the basic sequence part 119-151 were too small to be reliably identified. Interestingly, the fragment corresponding to positions 34-48 comprising the active site selenocysteine was not detected either. With these exceptions, however, the MALDI-TOF spectra

unequivocally complied with the PHGPx sequence and thus proved the presence of PHGPx in spots 1-7. On a thicker 2D-gel developed with a non-linear gradient from pH 3-10 also five distinct spots were detected in the 20 kDa region. In this experiments the presence of PHGPx was verified by microsequencing of major tryptic peptides (not shown). Again the spots representing PHGPx were the most prominent ones present in the gel.

The spots 1-6 of Fig. 2 a proved to be essentially homogeneous. As is exemplified in Fig. 2 b, the fragments yielding MALDI-TOF signals of significant intensities could be attributed to PHGPx. Only in the minor spot 7 a trace of impurity was detected, which according to the masses of fragments was tentatively identified as a mitochondrial contaminant (subunit of the T cell receptor variable region; acc. no. 228109). Based on integrated stain intensities of the individual spots those representing PHGPx amounted to about 50% of the capsule material. Most of the minor components (see Fig. 2 a) are likely derived from mitochondria included in the capsule preparation but are not necessarily constituents of the capsule, which is believed to be built up by apposition of extramitochondrial proteins onto the outer mitochondrial membrane. In other gels further proteins of mitochondrial origin like the mitochondrial glutathione S-transferase subunit Yb-2 (acc. no. 121719) and an endothelin converting enzyme (acc. no. 1706564) could be identified by MALDI-TOF or micro-sequencing (not shown). The only non-mitochondrial component identified was the "outer dense fiber protein", a cystine-rich structural sperm protein, which is associated with the helix of mitochondria in the sperm midpiece but also extends into the *flagellum* (7). In view of the nature of the additional proteins detected, the

PHGPx content of the actual mitochondrial capsule should substantially exceed the 50% observed by gel scanning.

Despite intense search, we could not detect any trace of the "sperm mitochondria-associated cysteine-rich protein ("SMCP") (7) in our capsule preparation. This cysteine- and proline-rich protein had for long been considered the selenoprotein accounting for the selenium content of the mitochondrial capsule in sperm (1,8,9). Cloning of the rat SMCP gene, however, revealed that it did not contain any in-frame TGA codon enabling selenocysteine incorporation (10). In mice, the three in-frame TGA codons proved to be upstream of the translation start (7). In developing mouse sperm SMCP stayed cytosolic up to states in which the mitochondrial capsule was already formed and only became superficially associated with the outer mitochondrial membranes of late spermatids and epididymal spermatozoa (7). SMCP thus is not necessarily an integral part of the mitochondrial capsule nor it is a selenoprotein. Instead, the "mitochondrial capsule selenoprotein (MCS)", as SMCP was originally referred to (1,7-10), is indeed PHGPx.

The chemical modifications of PHGPx leading to distinct differences in charge and apparent MW could not be reliably elucidated. Sequencing revealed an identical N-terminus of the size isomers starting with ASRDDWRCAR, i.e. a sequence either corresponging to the originally proposed translation start (11) after cleavage of the first two residues or derived from a possible pre-PHGPx (12) after processing of a mitochondrial leader peptide (13). Tryptic fragments extending towards the C-terminus up to position 164 were consistently observed also with the faster migrating specimen (Fig. 2 c) which leaves little room to explain an apparent MW difference of 1 to 1.5 kDa. As to

the charge isomers, it may be recalled that a potential phophorylation had been inferred from early attempts to sequence pig heart PHGPx (14). The assignment of masses to possibly phosphorylated tryptic peptides, however, remained equivocal. Certainly, more trivial events such as deaminations of Gln and Asn residues, C-terminal degradation, oxidation of the active site selenium, or its elimination might have contributed to the charge heterogeneity.

PHGPx as the major component of the sperm mitochondrial capsule had so far escaped attention, since as such it is enzymatically inactive, as it generally is in mature spermatozoa prepared from the tail of the epididymis (Tab. 1). It is neither reactivated by glutathione in the low millimolar range as used under conventional test conditions. High concentrations of thiols (0.1 M 2-mercaptoethanol or dithiothreitol), which in the presence of guanidine fully dissolve the capsule, regenerate a significant PHGPx activity, as measured after elimination of denaturating and reducing agents (Tab. 1). In fact, the specific activities thus obtained from mitochondrial capsules exceed, by a factor of 20, the highest values ever measured, i.e. in spermatogenic cells. Nevertheless, this extreme PHGPx activity is still low compared to its content in PHGPx protein. Based on the specific activity of pure PHGPx, the reactivated enzyme would be equivalent to less than 3% of the capsule protein, whereas the 2D-electrophoresis suggests a PHGPx protein content of at least 50%. It is worth noting that the same reductive procedure does not increase the specific activity of PHGPx in spermatogenic cells from testicular tubules (Tab. 1). The switch of PHGPx from a soluble active enzyme to an enzymatically inactive structural protein thus occurs during final differentiation of spermatozoa.

The alternate roles of PHGPx, being either a glutathione-dependent hydroperoxide reductase or a structural protein, are not necessarily unrelated. One of the features common to all glutathione peroxidases is a selenocysteine residue which together with a tryptophan and a glutamine residue forms a catalytic triad (15,16). Therein the selenol group of the selenocysteine residue is dissociated and highly activated by hydrogen bonding to reduce hydroperoxides with high rate constants. The reaction product, a selenenic acid derivative, R-SeOH, will readily react with thiols, e.g. GSH, to form an intermediate with a selenadisulfide bridge between enzyme and substrate, R-Se-S-G, from which the ground state enzyme can be regenerated by a second GSH. PHGPx is unique among the glutathione peroxidases in several respects: i) It usually is monomeric having its active site freely accessible at the surface; this facilitates interaction with bulky substrates. ii) Arginine residues surrounding the active site and specifically binding glutathione in most types of glutathione peroxidases are lacking in PHGPx (16); correspondingly, its specificity for the reducing substrate is less pronounced (16). It therefore can be envisaged that oxidized PHGPx may form diselenide or selenadisulfide bridges with exposed SeH or SH groups of proteins (16) including PHGPx itself, and this process, possibly followed by SH/SS, SH/SeS, or SH/SeSe exchange reactions, will create cross-linked protein aggregates. This ability of PHGPx might become particularly important if cells are exposed to hydroperoxides at extremely low concentration of glutathione, as is documented for late states of spermatogenesis (17-20). Fig. 3 is to mimick the oxidative events occurring during sperm maturation. Short term exposure of soluble proteins derived from spermatogenic cells to moderate H_2O_2 concentrations in the absence of GSH yields a variety of PHGPx-containing high molecular weight aggregates. Undoubtedly, therefore, PHGPx, by means of its intrinsic enzymatic potential, can

catalyse oxidative protein aggregation using protein thiols as alternate substrates. During sperm maturation, PHGPx thereby transforms itself into an enzymatically inactivated structural protein. This view, however, is not to imply that PHGPx could not depend on additional proteins when building up the highly organized architecture of the spermatozoal midpiece.

Our findings require a fundamental reconsideration of the role of selenium in male fertilty. The intriguing predominance of the selenoprotein PHGPx in the male reproductive system has so far been believed to reflect the necessity to shield germ line cells from oxidative damage by hydroperoxides and reactive oxygen species derived therefrom (11,17,21,22). This concept still merits attention with regard to the mutagenic potential of hydroperoxides and probably holds true for the early phases of spermatogenesis where PHGPx is still present as an active peroxidase (6,21). At this stage related activities reported for PHGPx or other glutathione peroxidases, e.g. silencing lipoxygenases (23), dampening the activation of NFκB (24) or inhibiting apoptosis (25), may also be relevant. In later stages of spermatogenesis characterized by a shift of the redox status resulting in loss of GSH (18-20,26), the ability of PHGPx to use protein thiols as alternate substrates opens up new perspectives of redox regulation which remain to be explored. In the mature spermatozoon PHGPx has experienced a pronounced metamorphosis now being a major constituent of the keratinuous material embedding the mitochondrial helix. It appears revealing that precisely this architectural pecularity in the midpiece of spermatozoa shows gross structural alterations in selenium deficiency. We therefore assume that the mechanical instability of the midpiece observed in selenium deficiency is a consequence of an impaired PHGPx biosynthesis. This view implies that it

is not the antioxidant capacity of PHGPx which is crucial for male fertility but its ability to utilize hydroperoxides to build an indispensable structural element of the spermatozoon.

As regards sreening, i.e. High Throughput Screening for a target molecule such as an inhibitor, reference can be made to for example Stahl in BioTec / Labortechnik, (2) (1998) 34 and Brecht et al. in BioTec / Labor-technik, (3) (1998) 26.

Methods

Preparation of rat spermatozoa, tubular cells and mitochondrial capsule

Spermatozoa of four month old Wistar rats (about 300 grams of body weight) were collected by squeezing *cauda epididymis* and *vas deferens* in phosphate buffer saline (PBS) and by centrifugating at 600 x g for 10 minutes. Cell and sperm pellets were layered on a discontinous 45%, 70% and 95% Percoll gradient and centrifugated at 300 x g for 20 min. Spermatogenic cells stacked on top of the gradient, while spermatozoa separated into the 70% Percoll layer. Cells from seminiferous epithelium were prepared as follows (26): testes were deprived of *albuginea*, seminiferous tubules were cut into small pieces in PBS containing 0.250 mg/ml collagenase, and incubated twice 25 °C for 15 min. Cells then were filtered through a stainless steel screen (140 μm pore), washed in PBS and centrifugated at 300 x g for 10 min. Sperm mitochondrial capsule was prepared according to Calvin et al.(1): sperms were resuspended in 0.05 M Tris - HCl pH 8.0 at the concentration of 10^6 cells/ml and treated with trypsin (0.2 mg/ml) for 10 minutes. After stopping the protease action with trypsin inhibitor (0.5 mg/ml) and SDS (10 mg/ml) sperms were centrifugated at 1,500 x g for 10 minutes. Pellets were resuspended in 0.05 M Tris - HCl, pH 8.5 containing 1% sodium dodecyl sulphate (SDS), and 0.2 mM DTT and kept under continuous stirring for 30 minutes. Following centrifugation at 4,500 x g for 15 min, the resulting supernatant was layered on a 1.6 M sucrose cushion. After centrifugation for 20 min at 18,000 x g in a swinging rotor, sperm capsules were collected as a band at the top of the sucrose cushion, washed in Tris – HCl, pH 8.0 and spun at 140,000 x g.

1D-electrophoresis and Western blotting

Electrophoresis was performed according to Laemmli under either reducing (+ 2-mercaptoethanol) or non-reducing conditions. Proteins were blotted onto nitrocellulose, probed with an antigen-purified rabbit antibody raised against pig heart PHGPx and detected by biotinylated anti rabbit IgG and streptoavidin alkaline phosphatase complex.

2D-electrophoresis

100µg of the mitochondrial capsule material was dissolved in 400 µl of a solution containing of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 20 mM Tris base and 0.5% IPG buffer (Pharmacia) and focused in an IPG-phor (Pharmacia) at 20°C by stepwise increasing voltage up to 5000 V but not exceeding a current of 30 µA per IPG strip. The pH gradient was non-linear from 3-10 or linear from 3-10 or 6-11. The focussed IPG strips were then equilibrated for SDS electrophoresis (10 min each) with a solution containing 60 mM DTT in 6 M urea, 30% glycerol, 0.05 M Tris-HCl buffer pH 8.8 and in the same buffer where DTT was substituted by 250 mM iodoacetamide. After SDS-electrophoresis (12% polyacrylamide) the gels were stained with Coomassie.

Protein identification

Coomassie-stained spots were cut out from the gels, neutralized with $(NH_4)HCO_3$, destained with 400 µl 50% acetonitrile/10 mM $(NH_4)HCO_3$ and dried in a Speed Vac Concentrator. Protein digestion was done overnight using 2 ng/µl sequencing grade trypsin (Promega) in 50 mM $(NH_4)HCO_3$ (Boehringer, Mannheim). The resulting peptides

were extracted twice with 60% acetonitrile / 40% H_2O / 0.1% TFA. Extracts were combined and lyophilized in the Speed Vac Concentrator. Peptide digests were desalted on small RP18-columns, eluted with saturated α-hydroxy-4-cyano-cinnamic acid and loaded directly onto the MALDI target (27). Reflectron MALDI mass spectra were recorded on a Reflex™ MALDI/TOF-mass spectrometer (Bruker-Franzen-Analytik, Bremen). The ions were excellerated at 20 kV and reflected at 21.3 kV. Spectra were externally calibrated using the monoisotopic MH^+ ion from two peptide standards. 100-200 laser shots were summed up for a single mass spectrum. Mass identifcation was performed with MS-Fit (http://falcon.ludwig.ucl.ac.uk/ucsfhtml/msfit.htm).

Alternatively, protein spots from 1.5 mm 2D-gels were digested with modified trypsin (Promega, sequencing grade) in 25 mM $(NH_4)HCO_3$ overnight at 37°C. The digests were extracted twice and dried as before and reconstituted in 10µl water. Peptides were separated on a reversed-phase capillary column (0.5 mm x 150 mm) with a gradient of acetonitrile in 0.1% formic acid / 4 mM ammonium acetate at a flow rate of 5µl/min and collected manually. Aliquots of 5 µl were spotted onto Biobrene-treated glass fiber filters and sequenced on an Applied Biosystems 494A sequencer with standard pulsed-liquid cycles. Before N-terminal sequencing, proteins were blotted from polyacrylamide gels onto PVDF membranes for 16 h at pH 8.3 (25 mM Tris-HCl, 192 mM glycine) and 100 mA (30 V).

When applicable, PHGPx was also identified by activity measurement according to (28) using the specific substrate phosphatidylcholine hydroperoxide.

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Figure 1 Presence of PHGPx in the mitochondrial capsule of spermatozoa.

a, Mitochondrial capsule prepared by trypsination and centrifugation according to (1) at 80,000 fold magnification. **b**, The same preparation as shown in **a**, but after exposure to 0.1 M 2-mercaptoethanol for 15 min at 4°C. Contamination of the capsule material by mitochondria is evident from the presence of mitochondrial ghosts. **c**, SDS gel electrophoresis of proteins extracted from capsule material (see Methods) by treatment with 0.1 M 2-mercaptoethanol, 0.1 M Tris-HCl, pH 7.5, and 8 M guanidine HCl. Left lane is stained with Coomassie, right lane demonstrates presence of PHGPx by Western blotting.

Figure 2 Analysis of the composition of the mitochondrial capsule of spermatozoa

a, 2D-electrophoresis of purified dissolved capsule material. Proteins were focused in a linear pH-gradient from 3 to 10 (horizontal direction), then reduced, amidocarboxymethylated, subjected to SDS-electrophoresis, and stained with Coomassie. MALDI-TOF analysis of the visible spots identified the following proteins (SwissProt data base): spot 1-7 PHGPx (MW 19 443; pI 8.27; acc. no. 544434); spots 8 and 9, outer dense fiber protein (MW 27351; pI 8.36; acc. no. P21769); spots 10 and 11, voltage-dependent anion channel-like protein (MW 31720; pI 7.44; acc. no. 540011); spot 12, "stress-activated protein kinase" (MW 48107; pI 5.65; acc. no. 493207); spot 13, glycerol-3-phosphate dehydrogenase (MW 76479; pI 5.86; acc. no. P35571).

b, MALDI-TOF spectrum (overview) of tryptic peptides obtained from PHGPx as found in spot 3. Abscissa, mass/charge ratio of the peptide fragments; ordinate, arbitrary units of

intensity; numbers at mass signals, identified peptides in the PHGPx sequence (see insert for position numbers); T, trypsin-derived fragments.

c, Compilation of tryptic PHGPx fragments identified in spots 1-7 by MALDI-TOF. Vertical lines designate potential tryptic cleavage sites. Dark blocks, identified typical cleavage products; shadowed blocks, masses resulting from incomplete cleavage or equivocally assignable to different fragments (e.g. 3-9 and 63-69).

Figure 3 Formation of PHGPx-containing aggregates from spermatogenetic cells by H_2O_2 in the absence of GSH. Spermatogenic cells were homogenised in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ml pepstatin A, 0.7 µg/ml leupeptin and 5mM 2-mercaptoethanol at pH 7.5 and 4°C. After centrifugation at 105,000 x g for 30 min, excess reagents were removed by gel permeation using NAP 5 columns equilibrated with 10mM Tris-HCl, 0.15 M NaCl, 1mM EDTA and 0.1% Triton X-100, pH 7.5. Immediately (t 0) and 15 min after (t 15) the addition of 75 µM H_2O_2 aliquots of the mixture (0.05 mg of protein) were withdrawn and subjected to electrophoresis under (a) reducing and (b) non reducing conditions. After blotting on nitrocellulose, PHGPx was detected by specific antibodies.

Figure 4

PHGPx specific activity in extracts (0.1 % Triton X-100 and 0.1 M 2-mercaptoethanol) of human sperm Correlation between this parameter and therapeutic approach in cases of couple infertility.

Figure 5

Relationship between PHGPx specific activity and number of "typical" sperms per milliliter of semen. "Typical" is a morfological parameter of sperm evaluation.
Note: artifacts by imprecise sampling are not ruled out.

Figure 6

Relationship between PHGPx specific activity and number of "fast" sperms per milliliter of semen. "Fast" is a parameter of sperm mobility.
Note: artifacts by imprecise sampling are not ruled out.

Table 1 PHGPx activity in spermatogenic cells, spermatozoa and sperm capsule. Effect of thiols.

Preparation	mU/mg protein [a, b]
Cells from seminiferous tubules	
5 mM 2-mercaptoethanol [c]	250 ± 10
100 mM 2-mercaptoethanol [c]	260 ± 10
Spermatozoa from tail of epididymis	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	3,140 ± 200
Mitochondrial capsule	
5 mM 2-mercaptoethanol [c]	undetectable
100 mM 2-mercaptoethanol [c]	5,600 ± 290

[a] One enzyme mU catalyzes the reduction of one nanomole of phosphatidylcholine hydroperoxide per minute at 37 °C in the presence of 3 mM GSH.

[b] Data are the mean and S. D. of five independent measurements.

[c] Solubilisation / reduction was carried out in 0.1 M Tris-HCl, 6 M guanidine-HCl, 0.5 µg/ ml pepstatin A, 0.7 µg/ml leupeptin and 2-mercaptoethanol as indicated at pH 7.5 and 4 °C for 10 min Low molecular weight compounds were removed before activity determination by a NAP 5 cartridge.

Unser Zeichen: 9808

Claims

1. Method for the determination of latent phospholipid hydroperoxide glutathione peroxidase (PHGPx) comprising the steps of
   a) obtaining a sperm sample,
   b) solubilizing the spermatozoa by using detergents and chaotropic agents and reactivating latent PHGPx by using high concentrations of thiols and
   c) determining enzymatic activity of reactivated latent PHGPx.

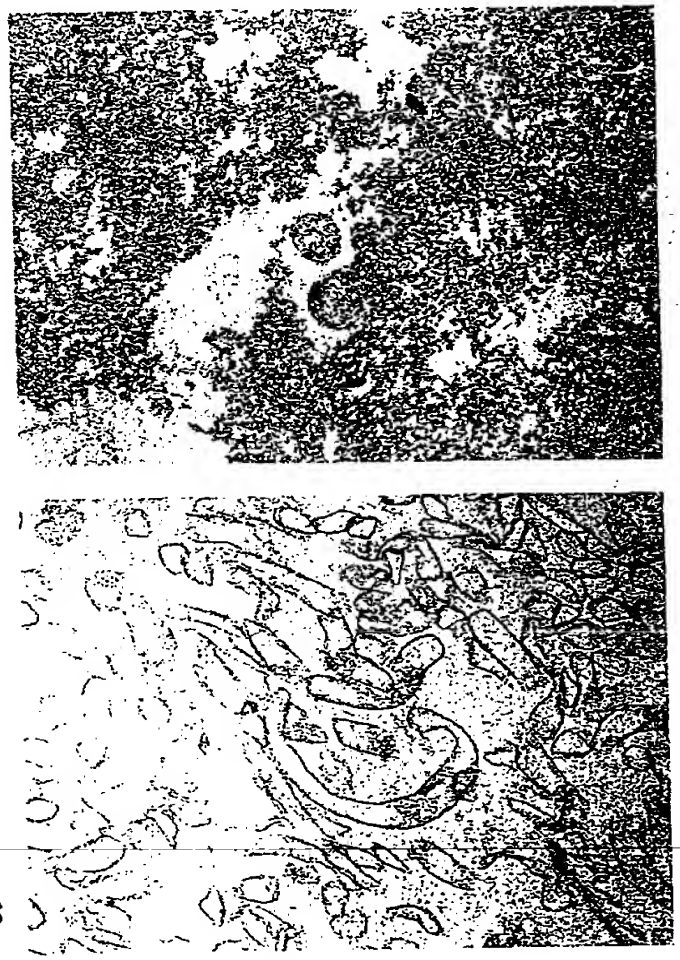
2. Method according to claim 1, wherein the chaotropic agent is 4 - 8 M guanidine chloride, 4 - 8 M guanidine thiocyanate or 5 - 8 M urea.

3. Method according to claim 1 or 2, wherein the thiol is 50 - 300 mM 2-mercaptoethanol, 25 - 300 mM dithiothreitol (DTT) or dithioerythritol (DTE).

4. Method according to any of the preceeding claims, wherein the sperm sample is from humans or life stock.

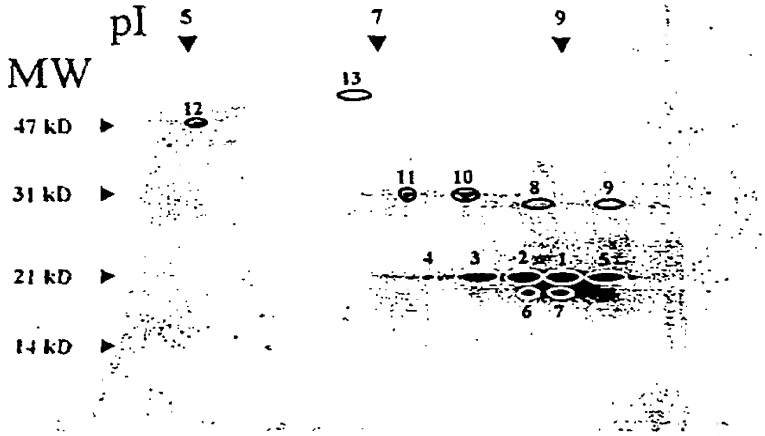
5. Use of a method of any of the preceeding claims in a method for predicting the fertilizing potential of spermatozoa in sperm samples.

Fig. 1

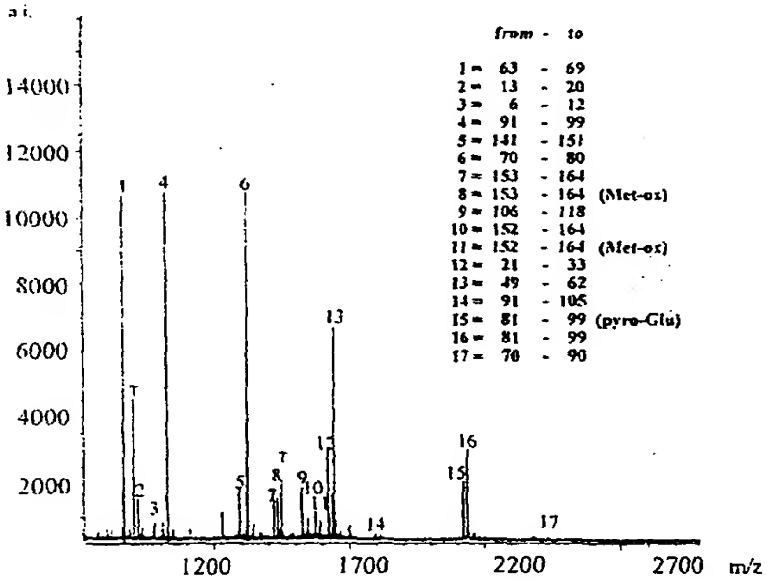


a)

Fig. 2


pI
5
7
9
MW
47 kD
31 kD
21 kD
14 kD
12
13
11
10
8
9
4
3
2
1
5
6
7

b)


a.i.
14000
12000
10000
8000
6000
4000
2000
1200
1700
2200
2700
m/z
from - to
1 = 63 - 69
2 = 13 - 20
3 = 6 - 12
4 = 91 - 99
5 = 141 - 151
6 = 70 - 80
7 = 153 - 164
8 = 153 - 164 (Met-ox)
9 = 106 - 118
10 = 152 - 164
11 = 152 - 164 (Met-ox)
12 = 21 - 33
13 = 49 - 62
14 = 91 - 105
15 = 81 - 99 (pyro-Glu)
16 = 81 - 99
17 = 70 - 90

c)

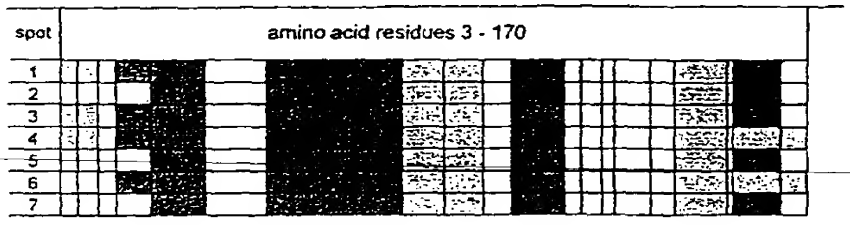

spot
amino acid residues 3 - 170
1
2
3
4
5
6
7

Fig. 3

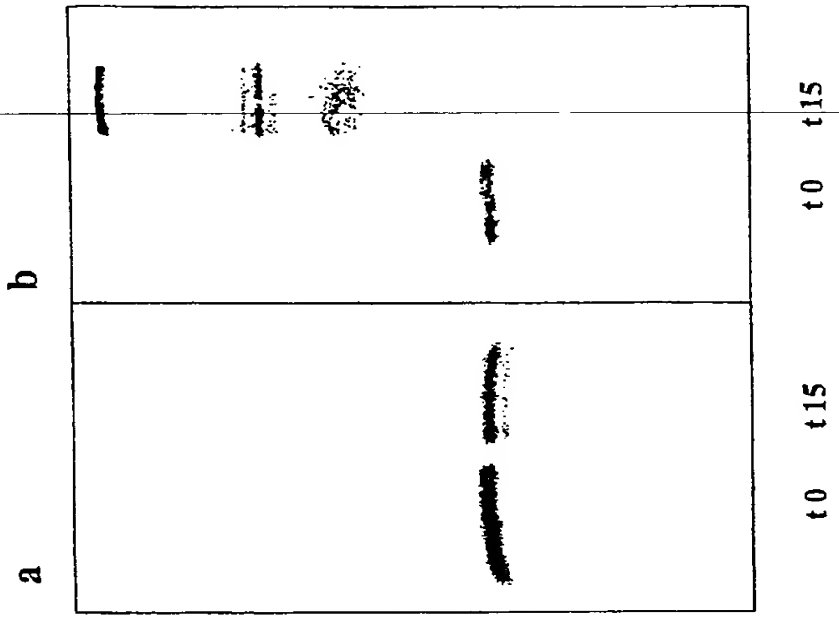

b
a
t0 t15
t0 t15

Fig. 4

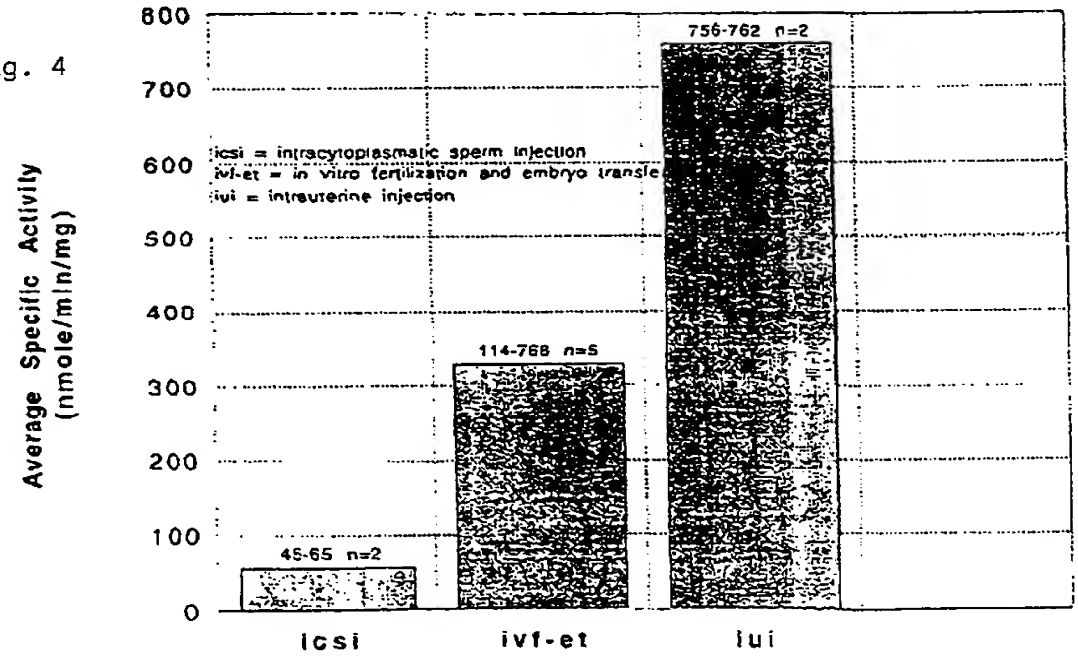

Average Specific Activity (nmole/min/mg)
800
700
600
500
400
300
200
100
0
icsi = intracytoplasmatic sperm injection
ivf-et = in vitro fertilization and embryo transfe
iui = intrauterine injection
45-65 n=2
114-768 n=5
756-762 n=2
icsi
ivf-et
iui

Fig. 5

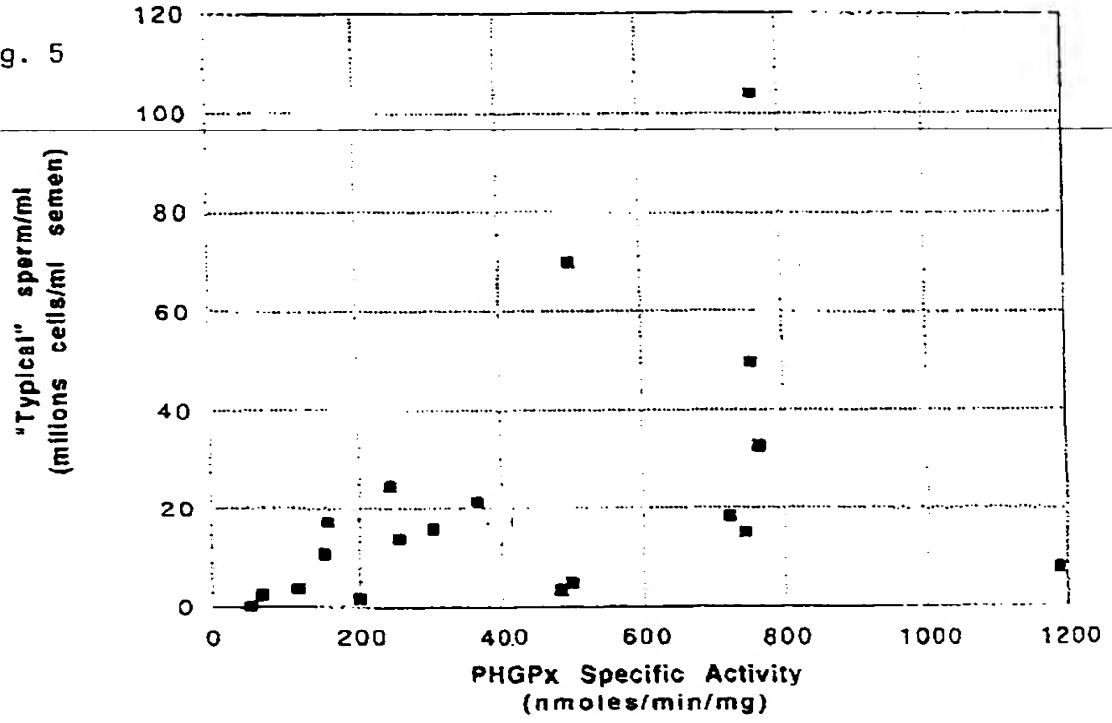
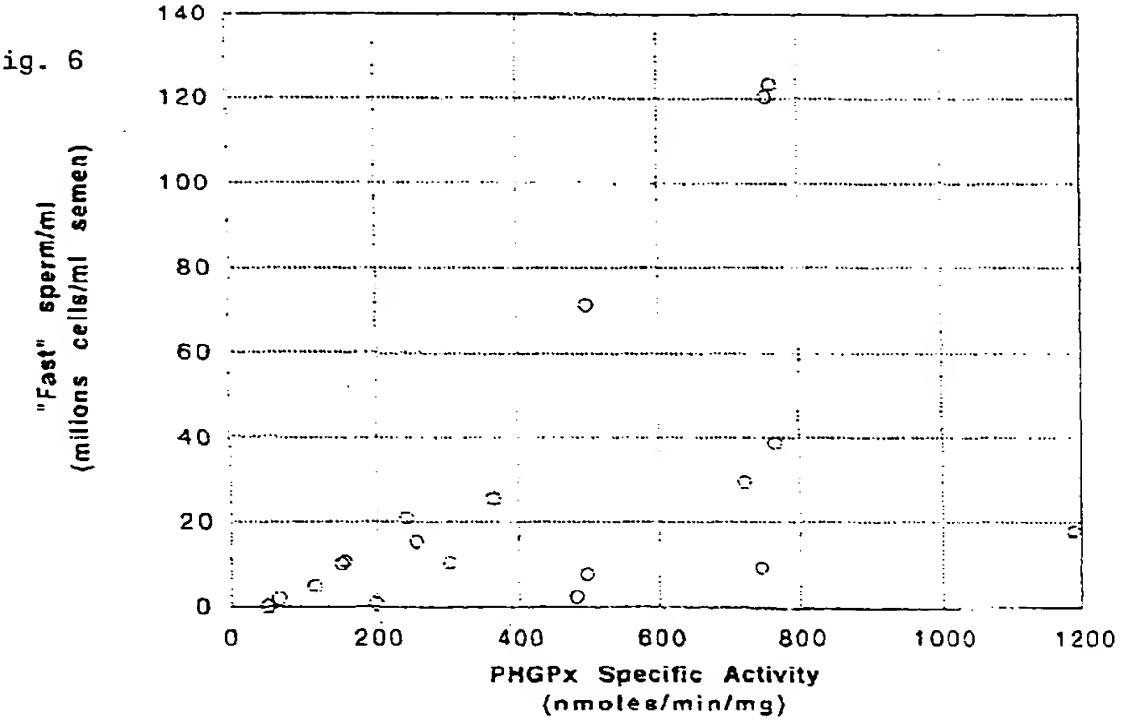

"Typical" sperm/ml
(millions cells/ml semen)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)

Fig. 6


"Fast" sperm/ml
(millions cells/ml semen)
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
PHGPx Specific Activity
(nmoles/min/mg)